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(57) Abstract

Oral anti-rabbies vaccines that comprise at least 100 μg of an isolated rabies glycoprotein G that is produced in eukaryotic cells and methods of immunizing mammals against rabies infection which comprise the step of orally administering to the mammal, such oral anti-rabies vaccine are disclosed. Chimeric proteins are disclosed. Orally active vaccines for immunizing a mammal against a pathogen infection comprising chimeric proteins and methods of using the same are disclosed. Oral anti-pathogen vaccines that comprise at least 100 μg of an isolated rabies glycoprotein G and either an isolated pathogen antigen or a peptide which comprises a neutralizing epitope of a pathogen antigen and methods of using the same are disclosed.

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Oral Vaccination of Mammals

Field of the Invention

The present invention relates to compositions for and methods of orally vaccinating mammals using isolated rabies glycoprotein G or chimeric proteins comprising fragments of rabies glycoprotein G and amino acid sequences which constitute epitopes against which an immunogenic response may be invoked.

Background of the Invention

Oral vaccination is not only economical and easy to administer, it also achieves wide distribution and is therefore particularly suitable for massive immunization. A few oral vaccines against viral diseases have been developed and proven to be effective, for example the oral live poliovirus vaccine (Sabin, A.B. and Boulger, L.R. (1973) *J. Biol. Stand.* 1:115). However, all oral vaccines currently in use or under clinical trail are either live-attenuated or live-recombinant viruses (Ada, G.L. (1990) *Semin. Virol.* 1:3; Blancou, J., *et al.* (1986) *Nature* 322:373; Rupprecht, C.E., *et al.* (1986) *Proc. Natl Acad. Sci USA* 83:7949). Live attenuated vaccines present risks relating to reversion to virulent forms of the pathogen. The use of live recombinant vaccines is associated with concerns and risks associated with the use of infectious agents.

Wildlife rabies exists in may countries and continues to present a major public health threat. Efforts to control wildlife rabies during the past decade in both Europe and North America have been directed toward oral vaccination of free-

ranging wildlife with live-attenuated rabies virus in bait (Wandeler, A.I. In: *The Natural History of Rabies*, 2nd ed. (Ed. Baer, G.M.). CRC Press, Boca Raton, Florida, p. 485-503, which is incorporated herein by reference). It has been proven to be very successful, especially in foxes (Baer, G.M. (1988) *Rev. Infect. Dis.* 10, S644; Schneider, L.G., *et al.* (1988) *Rev. Infect. Dis.* 10 S654). In the past few years, raccoon rabies has spread from the southeastern to the mid-atlantic region of the USA. Recently, a vaccinia recombinant virus containing the rabies glycoprotein (G) (Kieny, M.P..,*et al.* (1984) *Nature* 312:163) has been developed and demonstrated to be effective against rabies virus infection under laboratory conditions (Blancou, J., *et al.* (1986) *Nature* 322:373; Rupprecht, C.E., *et al.* (1986) *Proc. Natl Acad. Sci USA* 83:7949; Wiktor, T.J., *et al.*(1984) *Proc. Natl Acad. Sci. USA* 81: 7194). Field trials with this recombinant vaccine in foxes has resulted in large-scale elimination of rabies in vaccinated areas in Europe. Field trials with the same vaccine are currently underway in raccoons in the United States. Laboratory experiments have been reported which described effective vaccination of raccoons by oral administration of inactivated rabies virus PM strain (Rupprecht, C.E., *et al.* (1992) *J. Wildlife Diseases* 28(4):629-635).

The use of live-attenuated virus as a means of vaccinating animals requires that live infectious agents be placed in the environment. Such vaccination methods are accompanied by risks. There is a need for an oral vaccine against rabies infection which does not require the use of infectious agents.

Oral vaccines are additionally desirable as an alternative to injectable vaccines. For human vaccines, oral vaccines offer alternatives to other modes of administration which are associated with discomfort. Furthermore, there is a desire to eliminate the use of needle syringes whenever possible to reduce the transmission of any infectious agents between the vaccinated individual and the vaccine administrator. With respect to veterinary vaccines, the ease

of delivering vaccines in high volume using oral administration presents advantages over other modes of administering vaccines.

There remains a need to provide effective oral vaccines against rabies without the use of infectious agents or carriers. There remains a need to develop methods and compositions for oral vaccination against pathogens.

Summary of the Invention

The present invention relates to oral anti-rabies vaccines that comprise at least 100 μg of an isolated rabies glycoprotein G that is produced in eukaryotic cells together with a carrier suitable for oral administration.

The present invention relates to methods of immunizing mammals against rabies infection which comprise the step of orally administering to the mammal, an oral anti-rabies vaccine that comprises at least 100 μg of an isolated rabies glycoprotein G that is produced in eukaryotic cells.

The present invention relates to chimeric proteins that are modified rabies glycoprotein G proteins. The chimeric proteins comprise native rabies glycoprotein G amino acid sequence except with a 6-30 amino acid sequence substitution between amino acids 20 and 450 of the rabies glycoprotein G amino acid sequence. The 6-30 amino acid substitution comprises an amino acid sequence that constitutes a neutralizing epitope from a pathogen antigen.

The present invention relates to orally active vaccines for immunizing a mammal against a pathogen infection comprising chimeric proteins that are modified rabies glycoprotein G proteins. The chimeric proteins comprise native rabies glycoprotein G amino acid sequence except with a 6-30 amino acid sequence substitution between amino acids 20 and 450 of the rabies glycoprotein G amino acid sequence. The 6-30 amino acid substitution comprises an amino acid sequence that constitutes a neutralizing epitope from a pathogen antigen.

The present invention relates to methods of immunizing a mammal against a pathogen infection comprising the step of orally administering to the mammal a chimeric protein. The

chimeric protein comprises native rabies glycoprotein G amino acid sequence except with a 6-30 amino acid sequence substitution between amino acids 20 and 450 of the rabies glycoprotein G amino acid sequence. The 6-30 amino acid substitution comprises an amino acid sequence that constitutes a neutralizing epitope from a pathogen antigen.

The present invention relates to oral anti-pathogen vaccines that comprise at least 100 μg of an isolated rabies glycoprotein G that is produced in eukaryctic cells and at least 100 μg of either an isolated pathogen antigen or a peptide which comprises an amino acid sequence that constitutes a neutralizing epitope of the pathogen.

The present invention relates to a method of immunizing a mammal against a pathogen infection comprising the step of orally administering to the mammal, an oral anti-pathogen vaccine that comprises at least 100 μg of an isolated rabies glycoprotein G that is produced in eukaryotic cells and at least 100 μg of either an isolated pathogen antigen or a peptide which comprises an amino acid sequence that constitutes a neutralizing epitope of the pathogen.

Detailed Description of the Invention

According to some embodiments, the present invention provides an orally active anti-rabies vaccine that is an alternative to the recombinant vaccinia approach by providing a non-infectious vaccine which comprises isolated rabies virus G protein. The present invention provides oral immunization of mammals, particularly wildlife mammals, especially raccoons without the use of infectious agents. In some embodiments, the G protein of rabies virus is produced in eukaryotic cells. In some embodiments, the G protein of rabies virus is produced in insect cells using baculovirus vector. In some embodiments, the G protein of rabies virus Evelyn Rokitnicki Abelseth (ERA) strain is produced and used in the vaccine. In some embodiments, the G protein of rabies virus PM strain is produced and used in the vaccine. In some embodiments the G protein of rabies virus is produced in insect cells via a

baculovirus vector and used for oral vaccination of raccoons against rabies.

As used herein, the terms "rabies G protein" and rabies glycoprotein G" are used interchangeably.

Rabies G protein is provided in a vaccine that is suitable for oral administration isolated from viral particles. As used herein, the terms "isolated rabies glycoprotein G" is meant to refer to rabies G protein that is not part of an active or inactive virus. It is preferred that a composition comprising the isolated rabies glycoprotein G is essentially free from other rabies viral proteins.

Rabies G protein may be prepared routinely by recombinant DNA technology using well known techniques. The amino acid sequence and nucleotide sequence which encode the G protein are disclosed in Anilionis, A. *et al.*, (1981) *Nature* **294**:275-278 and Kieny, M.P. *et al.* (1984) *Nature* **312**:163, each of which is incorporated herein by reference. DNA molecules that comprise the coding sequence for rabies G protein can be obtained from natural sources or synthesized or otherwise constructed using widely available starting materials by routine methods. Rabies virus DNA is readily available and may alternatively be synthesized by well known techniques using widely available sequence information such as that in the above references. When the DNA that encodes the G protein is prepared synthetically, advantage can be taken of known codon preferences of the intended host where the DNA is to be expressed.

To produce G protein, one having ordinary skill in the art can, using well known techniques, obtain a DNA molecule encoding the G protein and insert that DNA molecule into a commercially available expression vector for use in well known expression systems. For example, the commercially available plasmid pYES2 (Invitrogen, San Diego, CA) may be used for production in *S. cerevisiae* strains of yeast. The commercially available MaxBac™ (Invitrogen, San Diego, CA) complete baculovirus expression system may be used for production in insect cells. The commercially available plasmid pcDNA I

(Invitrogen, San Diego, CA) may be used for production in mammalian cells such as Chinese Hamster Ovary cells.

One having ordinary skill in the art may use these or other commercially available expression vectors and systems or produce vectors using well known methods and readily available starting materials. Expression systems containing the requisite control sequences, such as promoters and polyadenylation signals, and preferably enhancers, are readily available and known in the art for a variety of hosts. *See e.g.*, Sambrook et al., *Molecular Cloning a Laboratory Manual*, Second Ed. Cold Spring Harbor Press (1989). Thus, the desired proteins can be prepared in both prokaryotic and eukaryotic systems, resulting in a spectrum of processed forms of the protein.

A wide variety of eukaryotic hosts are available for production of recombinant foreign proteins. Eukaryotic hosts may be transformed with expression systems which produce the desired protein directly, but more commonly signal sequences are provided to effect the secretion of the protein. Eukaryotic systems have the additional advantage that they are able to process introns which may occur in the genomic sequences encoding proteins of higher organisms. Eukaryotic systems also provide a variety of processing mechanisms which result in, for example, glycosylation, carboxy-terminal amidation, oxidation or derivatization of certain amino acid residues, conformational control, and so forth.

Commonly used eukaryotic systems include, but is not limited to, yeast, fungal cells, insect cells, mammalian cells, avian cells, and cells of higher plants. Suitable promoters are available which are compatible and operable for use in each of these host types as well as are termination sequences and enhancers, as e.g. the baculovirus polyhedron promoter. As above, promoters can be either constitutive or inducible. For example, in mammalian systems, the mouse metallothionene promoter can be induced by the addition of heavy metal ions.

The particulars for the construction of expression systems suitable for desired hosts are known to those in the

art., For recombinant production of the protein, the DNA encoding it is suitably ligated into the expression vector of choice and then used to transform the compatible host which is then cultured and maintained under conditions wherein expression of the foreign gene takes place. The protein of the present invention thus produced is recovered from the culture, either by lysing the cells or from the culture medium as appropriate and known to those in the art.

One having ordinary skill in the art can, using well known techniques, isolate the protein that is produced.

The G protein may be also isolated from natural sources (Dietzschold *et al.* 1978 *J. Gen. Virol.* **40**:131-135, which is incorporated herein by reference).

The G protein may be administered to an animal orally. At least 100 μg of protein are administered. A preferred range is at least 100 μg to 300 μg. More protein may be administered if available. In some embodiments, a preferred dose is about 300 μg.

In administering the vaccine of the present invention to animals in the wild, the protein is preferably encapsulated within wax or another innocuous encasing vehicle and mixed with bait. When the wild animal consumed the bait, the encased G protein is taken into the oral cavity with the bait. When chewed, the G protein is released and available for intraoral absorption including sublingual absorption. Ingested G protein may be absorbed in the intestine for example.

For administration to wildlife, the vaccine of the present invention comprises food which is mixed with the isolated G protein. The food serves as bait. The present invention is particularly useful to immunize raccoons against rabies infection. Preferred bait for raccoons includes ground fish. In some embodiments, 300 μg of G protein is encapsulated in small wax pellets which are then mixed with ground fish.

It is believed that, when administered orally, the G protein attaches to cells before entering the stomach. It is believed that, when administered orally, the G protein binds to receptors that are present on cells in the oral cavity or

pharynx. It is believed that upon attachment, such cells, which are involved in mucosal immunity, take up the attached protein. Attachment to such cells presents the protein to the immune system before it is degraded by the acids and enzymes in the gastrointestinal tract. The protein is presented as a target for an immune response.

The discovery that the G protein is useful as an orally active immunogen provides the means to delivery other immunogenic targets by oral administration. The G protein or portions thereof may be used as a vehicle or facilitator for the uptake of other orally administered peptide sequences. In particular, other immunogenic targets may be linked to G protein and delivered orally as a means to invoke an immune response against the immunogenic target. Thus, amino acid sequences that constitute neutralizing epitopes of a pathogen antigen may be inserted within the G protein itself to produce a chimeric protein or modified G protein. Alternatively, peptides and proteins which comprise amino acid sequences that constitute neutralizing epitopes of a pathogen antigen may be administered together with the G protein as a mixture. It is also within the scope of the invention that peptides and proteins which comprise amino acid sequences that constitute neutralizing epitopes of a pathogen antigen may be conjugated to the G protein by either peptide or non-peptide bonds. Linkage by peptide bonds produces a fusion protein while linkage by non-peptide bonds produces conjugated compositions including dimers, trimers and other multimers.

As used herein, the terms "amino acid sequences that constitute neutralizing epitopes of a pathogen antigen" and "neutralizing epitope" are used interchangeably and meant to refer to the amino acid sequences which induce a neutralizing immune response against the pathogen from which they are derived. Those having ordinary skill in the art can readily appreciate what is meant by "amino acid sequences that constitute neutralizing epitopes of a pathogen antigen" and can readily identify sequences from a pathogen antigen which induce an immune response against the pathogen which is capable of

neutralizing infection by the pathogen. One means to screen epitopes for their ability to induce neutralizing immune responses against pathogen infection is to administer a peptide to an animal in order to induce an immune response against the peptide. Serum is then withdrawn from the animal and mixed the serum with wild type pathogen. The mixture is added to a host of the pathogen such as cells permissive to infection by a viral pathogen. If the host is not infected by the mixture, the presence of neutralizing antibodies in the serum is indicated. Therefore, the peptide has an amino acid sequence that constitute neutralizing epitopes of a pathogen antigen.

Pathogens for which neutralizing epitopes may be identified from pathogenic antigens are selected from the group consisting of: viruses of the picornavirus family such as rhinoviruses, etheroviruses (including polioviruses, coxsackieviruses, echoviruses, and human enteroviruses such as hepatitis A virus) and apthoviruses; viruses of the calcivirus family; viruses of the togavirus family; viruses of the flariviridue family such as dengue, yellow fever, Japanese encephalitis, St. Louis encephalitis and tick borne encephalitis viruses; hepatitis C virus; viruses of the coronavirus family such as feline infectious peritonitis virus, feline enteric coronavirus, canine coronavirus, human respiratory coronaviruses; viruses of the rhabdovirus Family such as rabies virus; viruses of the filoviridue family such as Ebola virus; viruses of the paramyxovirus family such as paramyxoviruses (including Mumps virus), morbillivirus (including measles, canine distemper) and pneuminvirus (including respiratory syncytial virus); viruses of the orthomyxovirus family such as influenza viruses; viruses of the bungavirus family; viruses of the arenavirus family; viruses of the reovirus family such as reoviruses and rotaviruses; viruses of the retrovirus family including oncoriviruses (such as feline leukemia virus, HTLVI and HTLVII) and lentiviruses (including human immunodeficiency virus and feline immunodeficiency virus); viruses of the papovavirus family such as polyomaviruses and papillomavirus; viruses of the adenovirus

family; viruses of the parvovirus family; viruses of the herpesvirus family such as HSVI, HSVII, pseudorabies virus, cytomegalovirus and EBV; viruses of the poxvirus family; bacterial pathogens including pathogenic gram-positive cocci, pathogenic gram-negative cocci, pathogenic enteric gram-negative bacilli, pathogenic anaerobic bacteria, and pathogenic spirochetes, and pathogenic protozoans such as malaria.

According to some aspects of the invention, neutralizing epitopes from non-rabies pathogens are inserted into the rabies glycoprotein G in place of rabies glycoprotein G amino acid sequences. Thus, chimeric proteins are formed which are essentially rabies glycoprotein G but which contain neutralizing epitopes from a pathogen antigen inserted within the rabies glycoprotein G protein in place of native rabies glycoprotein sequences. The rabies glycoprotein G sequences provide the necessary sequences to render the chimeric protein orally active. The amino acid sequences that constitute neutralizing epitopes of a pathogen antigen present a target for an immune response which will protect the vaccinated individual from infection by the pathogen.

Those having ordinary skill in the art can readily engineer the nucleic acid sequence that encodes G protein to encode a chimeric protein according to the present invention by routine molecular biology techniques. The amino acid sequence and nucleotide sequence which encode the G protein are disclosed in Anilionis, A. *et al.*, (1981) *Nature* 294:275-278 and Kieny, M.P. *et al.* (1984) *Nature* 312:163, each of which is incorporated herein by reference. Using nucleic acids molecules that encode the G protein as starting material, nucleotides sequences that encode the neutralizing epitopes from other pathogens may be inserted into the G protein coding sequence in place of native G protein nucleotide sequences. The resulting nucleic acid molecule, which encodes a chimeric protein can be used to produce the chimeric protein in well known expression systems such as those which are described above as useful for expressing G protein. The chimeric protein may be routinely isolated for preparation and formulation as an

orally administered pharmaceutical. The isclation, formulation and administration protocols which are described above for isolating, formulating and orally administering the anti-rabies G protein vaccine can be used to in the isolation, formulation and orally administering protocols for anti-pathogen vaccines using the chimeric proteins described herein.

Some embodiments of the present invention relate to compositions for and methods of immunizing a mammal against a pathogen infection comprising the step of orally administering to the mammal a chimeric protein which is a modified rabies glycoprotein G. The chimeric protein contains insertions of 6-30 amino acids into rabies G protein. The inserted sequences comprise an amino acid sequence that constitutes a neutralizing epitope of the pathogen. The insertions are made in place of deletions from the rabies glycoprotein G sequence between amino acids 20 and 450 of rabies glycoprotein G. In some embodiments, the insertions are made in place of deletions from the rabies glycoprotein G ERA strain. In some embodiments, the insertions are made in place of deletions from the rabies glycoprotein G PM strain. In some embodiments, pharmaceutical compositions are formulated to contain at least 100 μg of the chimeric protein. In methods of the invention, the pharmaceutical composition which contains at least 100 μg of the chimeric protein is administered orally. In some embodiments, pharmaceutical compositions are formulated to contain at least 300 μg of the chimeric protein. In methods of the invention, the pharmaceutical composition which contains at least 300 μg of the chimeric protein is administered orally. In some preferred embodiments, the mammal to be vaccinated is a human. In some preferred embodiments, the pathogen to be targeted is selected form the group set out above as the list of pathogens from which neutralizing epitopes of antigens may be identified. In some preferred embodiments, two or more neutralizing epitopes from the pathogen to be targeted are inserted into the G protein sequence. In some preferred embodiments, two or more neutralizing epitopes from two or more pathogens, respectively, to be targeted are inserted into the

G protein sequence. In some preferred embodiments, the amino acid sequence that constitutes a neutralizing epitope of the pathogen is 6-25 amino acids. In some preferred embodiments, the amino acid sequence that constitutes a neutralizing epitope of the pathogen is 8-20 amino acids. In some preferred embodiments, the amino acid sequence that constitutes a neutralizing epitope of the pathogen is 9-15 amino acids. In some preferred embodiments, the amino acid sequence that constitutes a neutralizing epitope of the pathogen is 9 amino acids. In some embodiments, the neutralizing epitope is a 6-9 amino acid sequence which is within a 9 amino acid sequence inserted into rabies glycoprotein G at positions 191-198 in place of the rabies G protein sequences. In some preferred embodiments, the amino acid sequence that constitutes a neutralizing epitope of the pathogen is 20-25 amino acids. In some preferred embodiments, the amino acid sequence that constitutes a neutralizing epitope of the pathogen is 23 amino acids. In some preferred embodiments, the amino acid sequence that constitutes a neutralizing epitope of the pathogen is 20-23 amino acids which is inserted within a 23 amino acid sequences inserted into rabies glycoprotein G at positions 253-275.

In some preferred embodiments, the amino acid sequence that constitutes a neutralizing epitope of the pathogen is inserted in place of deletions from the rabies glycoprotein G sequence between amino acids 175 and 225 of the rabies glycoprotein G. In some preferred embodiments, the amino acid sequence that constitutes a neutralizing epitope of the pathogen is inserted in place of deletions from the rabies glycoprotein G sequence between amino acids 185 and 215 of the rabies glycoprotein G. In some preferred embodiments, the amino acid sequence that constitutes a neutralizing epitope of the pathogen is inserted in place of deletions from the rabies glycoprotein G sequence between amino acids 190 and 205 of the rabies glycoprotein G. In some preferred embodiments, the amino acid sequence that constitutes a neutralizing epitope of the pathogen is inserted in place of deletions from the rabies

glycoprotein G sequence between amino acids 191 and 198 of the rabies glycoprotein G. In some preferred embodiments, the amino acid sequence that constitutes a neutralizing epitope of the pathogen is inserted in place of deletions from the rabies glycoprotein G sequence between amino acids 240 and 295 of the rabies glycoprotein G. In some preferred embodiments, the amino acid sequence that constitutes a neutralizing epitope of the pathogen is inserted in place of deletions from the rabies glycoprotein G sequence between amino acids 245 and 290 of the rabies glycoprotein G. In some preferred embodiments, the amino acid sequence that constitutes a neutralizing epitope of the pathogen is inserted in place of deletions from the rabies glycoprotein G sequence between amino acids 250 and 280 of the rabies glycoprotein G. In some preferred embodiments, the amino acid sequence that constitutes a neutralizing epitope of the pathogen is inserted in place of deletions from the rabies glycoprotein G sequence between amino acids 253 and 275 of the rabies glycoprotein G.

In some preferred embodiments, the amino acid sequence that constitutes a neutralizing epitope of the pathogen is 8-25 amino acids and inserted in place of deletions from the rabies glycoprotein G sequence between amino acids 175 and 225 of the rabies glycoprotein G. In some preferred embodiments, the amino acid sequence that constitutes a neutralizing epitope of the pathogen is 9-15 amino acids and inserted in place of deletions from the rabies glycoprotein G sequence between amino acids 185 and 215 of the rabies glycoprotein G. In some preferred embodiments, the amino acid sequence that constitutes a neutralizing epitope of the pathogen is 9-15 amino acids and inserted in place of deletions from the rabies glycoprotein G sequence between amino acids 190 and 205 of the rabies glycoprotein G. In some preferred embodiments, the amino acid sequence that constitutes a neutralizing epitope of the pathogen is 9 amino acids and inserted in place of deletions from the rabies glycoprotein G sequence between amino acids 191 and 198 of the rabies glycoprotein G. In some preferred embodiments, the amino acid sequence that constitutes a

neutralizing epitope of the pathogen is 20-25 amino acids and inserted in place of deletions from the rabies glycoprotein G sequence between amino acids 240 and 295 of the rabies glycoprotein G. In some preferred embodiments, the amino acid sequence that constitutes a neutralizing epitope of the pathogen is 20-25 amino acids and inserted in place of deletions from the rabies glycoprotein G sequence between amino acids 245 and 290 of the rabies glycoprotein G. In some preferred embodiments, the amino acid sequence that constitutes a neutralizing epitope of the pathogen is 20-25 amino acids and inserted in place of deletions from the rabies glycoprotein G sequence between amino acids 250 and 280 of the rabies glycoprotein G. In some preferred embodiments, the amino acid sequence that constitutes a neutralizing epitope of the pathogen is 23 amino acids and inserted in place of deletions from the rabies glycoprotein G sequence between amino acids 253 and 275 of the rabies glycoprotein G.

According to the present invention, chimeric proteins may be modified rabies glycoprotein G in which amino acid sequences which define neutralizing epitopes for non-rabies pathogens are inserted in place of G protein sequences. These substitutions are preferably amino acid for amino acid, that is a six amino acid sequence which constitutes a non-rabies neutralizing epitope is inserted into rabies G protein in place of six deleted sequences from rabies G protein. It is contemplated that inserts which contain additional amino acids may be inserted into deletion sites and that inserts which contain fewer amino acids may be inserted into deletion sites. It is also contemplated that portions of the G protein will be deleted without a corresponding insert form a non-rabies pathogen antigen.

In particular, according to some contemplated embodiments, truncated forms of the G protein are linked to amino acid sequences that constitutes a neutralizing epitope of the pathogen. Such fusion proteins contain the portion of the G protein which attaches to the receptor and renders the molecule orally active and an epitope against which an immune

response can be directed to protect against non-rabies infection. As used herein, the term "orally active fragment of G protein" refers to a fragment of the G protein which retains the unexpected ability to be taken up and invoke an immune response when orally administered. When administered orally, orally active fragments of the G protein are able to be presented as immunogenic targets against which an immune response is induced. In some contemplated embodiments, neutralizing epitopes form non-rabies pathogens are linked to orally active fragments of G protein. When administered orally, the portion of the chimeric protein which is an orally active fragment of G protein attaches to cells in the oral cavity and pharynx. An immune response is invoked against the non-rabies neutralizing epitope of the chimeric protein.

According to some aspects of the invention, compositions for and methods of immunizing a mammal against a pathogen infection are provided. The compositions orally administered to the mammal. The compositions comprise at least 100 μg of isolated rabies glycoprotein G produced in eukaryotic cells and at least 100 μg of a pathogen protein which comprises a neutralizing epitope. In some embodiments, the rabies glycoprotein G and the peptide or protein which comprises the neutralizing epitope of a non-rabies pathogen are unlinked and in some embodiments, the rabies glycoprotein G and the peptide or protein which comprises the neutralizing epitope of a non-rabies pathogen are linked by peptide or non-peptide bonds. When orally administered, the rabies glycoprotein G are taken up by the cells of the oral cavity and pharynx and the peptide or protein which comprises the neutralizing epitope of a non-rabies pathogen, whether linked or unlinked to the rabies G protein is similarly taken up. The amino acid sequences that constitute neutralizing epitopes of a pathogen antigen present a target for an immune response which will protect the vaccinated individual from infection by the pathogen.

The rabies glycoprotein G is described above. In some embodiments, the rabies glycoprotein G is from rabies ERA strain. Those having ordinary skill in the art can readily

produce peptides and proteins which comprise an amino acid sequences that constitutes neutralizing epitopes of a pathogen antigen. Such amino acid sequences, which induce an immune response against the pathogen which is capable of neutralizing infection by the pathogen, can be readily identified from a pathogen antigen. In some embodiments, the composition comprises a non-rabies pathogen antigen.

In some embodiments, pharmaceutical compositions are formulated to contain at least 100 μg of the rabies G protein and at least 100 μg of the peptide and protein which comprises an amino acid sequences that constitutes neutralizing epitopes of a pathogen antigen. In methods of the invention, the pharmaceutical composition which contains at least 100 μg of the rabies G protein and 100 μg of peptide and protein which comprises an amino acid sequences that constitutes neutralizing epitopes of a pathogen antigen is administered orally.

In some embodiments, pharmaceutical compositions are formulated to contain at least 300 μg of the rabies G protein and at least 100 μg of the peptide and protein which comprises an amino acid sequences that constitutes neutralizing epitopes of a pathogen antigen. In methods of the invention, the pharmaceutical composition which contains at least 300 μg of the rabies G protein and 100 μg of peptide and protein which comprises an amino acid sequences that constitutes neutralizing epitopes of a pathogen antigen is administered orally.

In some embodiments, pharmaceutical compositions are formulated to contain at least 100 μg of the rabies G protein and at least 300 μg of the peptide and protein which comprises an amino acid sequences that constitutes neutralizing epitopes of a pathogen antigen. In methods of the invention, the pharmaceutical composition which contains at least 100 μg of the rabies G protein and 300 μg of peptide and protein which comprises an amino acid sequences that constitutes neutralizing epitopes of a pathogen antigen is administered orally.

In some embodiments, pharmaceutical compositions are formulated to contain at least 300 μg of the rabies G protein and at least 300 μg of the peptide and protein which comprises

an amino acid sequences that constitutes neutralizing epitopes of a pathogen antigen. In methods of the invention, the pharmaceutical composition which contains at least 300 μg of the rabies G protein and 300 μg of peptide and protein which comprises an amino acid sequences that constitutes neutralizing epitopes of a pathogen antigen is administered orally.

The compositions and methods of the present invention can be applied to human medical and veterinary medical uses. The present invention relates to compositions and methods of immunizing mammals, particularly humans and bovine, ovine, porcine, equine, canine and feline species. In some preferred embodiments, the mammal to be vaccinated is a human.

In some preferred embodiments, the pathogen to be targeted is selected from the group consisting of those pathogens set out above.

In some embodiments, the peptide and protein which comprises an amino acid sequences that constitutes neutralizing epitopes of a pathogen antigen is linked to the rabies glycoprotein G. In some embodiments, the peptide and protein which comprises an amino acid sequences that constitutes neutralizing epitopes of a pathogen antigen is linked to the rabies glycoprotein G by peptide bonds. In some embodiments, the peptide and protein which comprises an amino acid sequences that constitutes neutralizing epitopes of a pathogen antigen is linked to the rabies glycoprotein G by non-peptide bonds. In some embodiments, the peptide and protein which comprises an amino acid sequences that constitutes neutralizing epitopes of a pathogen antigen is linked to the rabies glycoprotein G by disulfide bonds. In some embodiments, at least 2, and in some preferred embodiments, multiple copies of the peptide and protein which comprises an amino acid sequences that constitutes neutralizing epitopes of a pathogen antigen is linked to each rabies glycoprotein G molecule.

The compositions of the present invention are useful as components together with other vaccine compositions to form multivalent vaccines. Other components include other orally active vaccine products polio vaccines.

Examples

Example 1

The G protein gene of rabies virus ERA strain was recovered from plasmid ptg155pro8 (Kieny, M.P..,*et al.* (1984) *Nature* 312:163) by digestion with *Bgl*II restriction enzyme (Boehringer Mannheim Biochemicals) and subsequently cloned into the unique *Bam*HI site of baculovirus transfer vector pVL941 (Luckow,V.A. and Summers, M.D. (1898) *Virology* 170:31 which is incorporated herein by reference). A recombinant plasmid with the G gene in the correct orientation in relation to the polyhedron promoter was obtained and cotransfected with *Autographa californica* nuclear polyhidrosis virus (AcNPV) DNA into *Spodoptera Frugiperda* (Sf9) cells. A recombinant virus (BVLRG) was selected by limited dilution in combination with dot-blot hybridization and plaque purification, as described (Summers, M.D. and Smith, G.E. 1987 *Tex. Agric. Exp. St. Bull.* 1555:1 which is incorporated herein by reference).

To analyze the G protein expressed in the baculovirus system, Sf9 cells grown in a 24-well plate were mock-infected or infected with AcNPV or the recombinant baculoviruses BVLRG at multiplicity of infection of 10 plaque forming units per cell. At 47 h postinfection, the culture medium was removed and 200 µl methionine-free Grace's medium was added to each well. After incubation for 1 h, the medium was replaced with fresh methionine-free Grace's medium mixed with 30 µCi [^{35}S]methionine. After incubation for a further 4 h, cells in the wells were harvested, lysed with protein disruption buffer (2% sodium dodecyl sulphate, 10% glycerol, 5% β-mercaptoethanol, 62.5 mM Tris/HCl, 0.01% bromophenol blue, pH 6.8), and subjected to electrophoresis on a 10% polyacrylamide gel. The gel was then either dried under vacuum and autoradiographed or electrophoretically transferred to nitrocellulose for Western blot analysis. The membrane was blocked with 5% milk powder (Carnation) for 30 min and then incubated with 1/1000 dilution of rabbit anti-rabies G polyclonal antibodies for 1 h. The bound antibody was detected using a peroxidase-conjugated anti-rabbit IgG immunoglobulin

(BMB). The G protein expressed in Sf9 cells migrated slightly faster on the gels compared to the native G protein of ERA strain of rabies virus. It can also be seen from the pattern of the wild-type AcNPV that there is a protein of the wild-type virus that comigrates with the G protein expressed in Sf9 cells. After the proteins on the gel were transferred to nitrocellulose paper, only the G protein from BVLRG-infected cells and the G protein of rabies virus reacted with rabbit anti-G polyclonal antibodies.

Although the G protein expressed in insect cells reacted with the rabbit anti-G polyclonal antisera in the Western blot assay, it was uncertain whether or not the G protein expressed in cells retained its native structure. For this reason, a panel of 41 anti-G monoclonal antibodies (mAb) Wiktor, T.j. and Koprowski, H. (1978) *Proc. Natl Acad. Sci. USA* 75:3938; Bunschoten, H., *et al.* (1987) *J. Gen. Virol.* 70:291; Dietzschold, B., (1988) *Rev. Infect. Dis.* 10:S785), most of which recognize the native form of the G protein, were used for the antigenic analysis of the G protein expressed in Sf9 cells, in comparison with authentic G protein present in rabies virions, using an indirect immunofluorescence test. Sf9 cells and baby hamster kidney (BHK)-21 cells in Terasaki plates (Nunc) were infected respectively with BVLRG and ERA viruses at a multiplicity of infection of 10. After incubation for 24 h, cells were washed with phosphate-buffered saline, fixed with 80% cold ethanol and incubated with the 41 anti-G mAb. the reaction was detected by fluorescein-conjugated anti-mouse IgG (Cappel) and graded from negative (-) to brilliant fluorescence (++++). As summarized in Table 1, 33 mAb which reacted with the ERA virus-infected BHK-21 cells infected with ERA rabies virus or the Sf9 cells infected with BVLGR. These results indicate that the G protein expressed in insect cells retained its native structure.

Example 2

To test the potential for the G protein expressed in Sf9 cells as an oral vaccine in raccoons against lethal rabies virus infection, Sf9 cells were infected with BVLRG, harvested

at 60 h postinfection and lysed with either 1% Triton X-100 or 1% β-octylpyranoglucoside. The lysates were then centrifuged at 10 000 g for 20 min and the supernatants were recovered. The concentration of G protein in the supernatant was estimated by SDS-polyacrylamide gel electrophoresis using known amounts of purified rabies virion G protein as standard markers (data not shown). Eight raccoons were given 1 ml each *per os* of the lysates containing approximately 100 μg of G protein at day 0 and 200 μg cf G protein at 7 weeks. Animals were sedated with ketamine hydrochloride and bled by venipuncture on day 0 and weekly thereafter, as described in Rupprecht, C.E., *et al.* (1986) *Proc. Natl Acad. Sci USA* 83:79495. Serum neutralization antibody titers against rabies were determined using a modification of the rapid fluorescent focus-inhibition test, as described by Wiktor *et al.* 1983 *Dev. Biol. Stand.* 57:199. Vaccinated animals, as well as controls, were challenged with a street rabies virus and observed for three months after challenge for disease and deaths. Six of the seven immunized raccoons survived the challenge while all of the controls succumbed to rabies and dies within 30 days of challenge (Table 2).

The results indicate that approximately 300 μg of G protein expressed in insect cells in a crude form can stimulate protective immunity in raccoons against rabies when administered orally.

Example 3

The protective effect of oral administration of rabies glycoprotein G isolated from virus particles was determined as follows. The Fix Rabies Virus ERA (The Wistar Institute virus collection, Philadelphia, PA) was propagated on BHK-21 cell monolayers grown at 37°C minimum essential medium supplemented with 10% fetal calf serum. Cell cull supernatants were harvested and inactivated by beta propylactone (BPL) treatment (1:4000). The virus suspension was then concentrated and purified by zonal ultracentrifugation on a sucrose gradient. Each purified virus was suspended in phosphate-buffered saline and was adjusted to a protein concentration of 10 micrograms

per microliter. Inactivated virus was purified by adding an equal volume of 4% OGP-NT buffer and incubated for one hour at room temperature. The suspension was centrifuged on a 25% sucrose cushion (NaCl 0.5 M, OGP 2%) in an SW 50 rotor at 12,000 G for 100 minutes at 4°C. The despiculated viral particles were in the sediment. The supernatant was harvested and adjusted to 2.5% sucrose by adding NT buffer. A sucrose gradient was prepared in NaCl 0.5 M, OGP 2% with three layers: one ml at 60% (W/V), 8 ml of linear gradient 5 to 25% (W/V); and one ml at 3% (W/V). The glycoprotein suspension was deposited onto the sucrose gradient and centrifuged in a SW 41 rotor at 150,000 G for 36 hours at 4° centigrade. Fractions were harvested in quantities of 0.5 ml from the bottom of the tubes. Fractions which contain glycoproteins are identified. The glycoprotein solution is dialyzed against NT buffer overnight at 4°C and sterilized by filtration through a .22 μ membrane.

Animals used to test the protective capability of the viral protein consisted of rabies seronegative adult raccoons 1-5 years of age. Prior to handling, all animals were sedated by intramuscular administration of .5 to 1.0 ml of ketamine hydrochloride. Raccoons were given rabies viral G protein *per os* by needleless syringe: One group of raccoons consisting of six animals was sedated and administered a single 1.0 ml dose of 100 micrograms of ERA rabies G vaccine *per os*. A second group of raccoons received 1.0 ml of PBS by installation directly into the mouth as a control.

All animals remained healthy throughout the ninety day observation period. In the six raccoons administered 100 micrograms of ERA rabies G vaccine *per os*, four survived lethal intramuscular challenge on day 90 with 1 x 10^{55}MICLD$_{50}$/ml of street rabies virus strain MD5951 whereas all six controlled raccoons succumbed.

Example 4

The human immunodeficiency viruses (HIVs) are the etiologic agents for acquired immunodeficiency syndrome (AIDS). Several lines of evidence suggest that the induction of

neutralizing antibodies is a key factor in protective immunity against HIV. The envelope glycoprotein gp120 (or gp160) has been the major target for developing candidate vaccines against AIDS since gp120 recognizes the cellular receptor (CD4) on T lymphocytes and induces neutralizing antibodies. A principal neutralization determinant (PND) has been identified and is located in the third variable domain of the gp120.

Chimeric genes containing rabies G protein sequences and PND sequences of HIV-1 gp120 are constructed by replacing linear and/or discontinuous epitopes of rabies virus G protein with PND sequences of HIV-1 gp120. In some embodiments these chimeric proteins are expressed in mammalian cells via vaccinia virus vectors and in insect cells via baculovirus vectors.

Several lines of evidence suggest that the induction of neutralizing antibodies is a key factor in protective immunity against HIV. Individuals infected by HIV-1 usually develop antibodies that neutralize the virus *in vitro*, and neutralization titers seem to decrease with progressive disease, suggesting that the neutralizing antibodies may be protective. Protection experiments have indicated that protection in monkeys against HIV immunized with recombinant envelope proteins correlates with the titers of neutralizing antibodies at the time of challenge. Furthermore monoclonal antibodies which have been shown to neutralize HIVs *in vitro* can also protect chimpanzees against challenge infection *in vivo*. Since most of the neutralizing antibodies are elicited by the envelope protein, most efforts in HIV vaccine development to date have been focused on the envelope glycoprotein. The envelope of HIV-1 is known to contain only two proteins, the exterior surface glycoprotein of 120 kDa (gp120) and the transmembrane protein of 41 kDa (gp41), both of which are derived from a common precursor, gp160. It was shown that recombinant proteins or viruses, like the native proteins purified from HIV-infected cells, induced high levels of HIV-specific antibodies, some with neutralizing activity, in experimental animals or human volunteers. Immunization of monkeys with the recombinant proteins purified from CHO cells

have resulted in the induction of neutralizing antibodies and some of the immunized monkeys with high neutralizing antibody titers were protected against a challenge infection with homologous virus strain.

Most of the neutralizing antibodies elicited by recombinant envelope proteins in test animals have been mapped to the central portion of gp120 (amino acids 307 to 338) since these antibodies can be absorbed with synthetic peptides derived from this region. Furthermore, similar peptide was found to absorb substantial fractions of neutralizing antibodies in sera from HIV-1 infected people. All these peptides are within a disulfide-linked loop in the third variable domain (V3 loop) of gp120. This principal neutralizing determinant (PND) is one of the more variable regions of the envelope and differs by as much as 50% among HIV isolates which explains why the neutralizing antibodies stimulated by recombinant envelope proteins are type-specific. Neutralization antibodies elicited by synthetic peptides derived from PND have also been shown to be strain-specific. However, recent sequence analysis of many HIV isolates from North America and Europe revealed that sequence in the central portion of V3 loop, GPGRAF (SEQ ID NO: 1), is relatively conserved although the sequences flanking the GPGRAF (SEQ ID NO: 1) regions in these isolates may be different. Over 65% of HIV-1 positive sera reacted with peptides containing this sequence. Furthermore, antisera from rabbits immunized with a peptide containing the GPGRAF (SEQ ID NO: 1) residues neutralized divergent isolates such as III_B and MN. Monoclonal antibodies selected against the PND have been shown to neutralize HIV infectivity *in vitro* and to protect chimpanzees against HIV challenge infection *in vivo*.

The data summarized above demonstrate that subunit vaccines such as the envelope proteins (gp120 and gp160) or peptides derived from it can stimulate the production of neutralizing antibodies in immunized animals or humans. The results obtained from immunized monkeys also show that vaccination with these subunit vaccines can also protect

against infection if the neutralizing antibody titers are high enough at the time of challenge. The peptide vaccines derived from PND may have some advantages over the entire envelope protein since some antibodies to the envelope protein but not to the PND have been shown to enhance HIV infectivity *in vitro* although this has not been reported *in vivo*

More research should be directed to develop vaccines to induce local mucosal immunity. One way to stimulate local immune responses is by oral immunization. Oral vaccination not only stimulates IgA precursor B cells in the gut-associated lymphoid tissues but also leads to the dissemination of B and T cells to other mucosal effector tissues including genital tracts for subsequent antigen-specific S-IgA antibody production.

In one embodiment of the present invention rabies virus glycoprotein is used as a vehicle to target HIV antigens to the pregastric mucous membrane since rabies virus G protein is a superb oral immunogen and can induce systemic immune responses when given orally. Although the local immune responses to the rabies virus G protein have not been investigated, it is believed that it can also stimulate local immune responses. A linear neutralizing epitope has been defined on the G protein and immunization with a peptide derived from this epitope resulted in the production of neutralizing antibodies and protected mice against lethal challenge, which provide the sites to engineer HIV epitopes. We have also identified an epitope at antigenic site II of rabies virus G protein by computer modeling. Epitopes of rabies G protein are replaced with HIV PND epitopes from MN-like strain since MN-like strains are the most prevalent in both North America and Europe and peptides derived from PND of these strains can induce broadly neutralizing antibodies. The chimeric protein will be expressed either in insect cells via baculovirus or in mammalian cells by vaccinia viruses and then studied for its antigenicity and immunogenicity.

To confirm that rabies virus glycoprotein is a good oral immunogen, we have expressed the glycoprotein in a

baculovirus expression system and studied its oral immunogenicity. To express rabies virus G protein, the G protein gene of rabies virus ERA strain was recovered from plasmid ptg 155pro8 by digestion with BgIII restriction enzyme and cloned into the unique BamHI site of a baculovirus transfer vector pVL941. After cotransfection of the recombinant plasmid and wide type baculovirus DNA into Sf9 cells, the recombinant baculovirus containing the rabies virus G protein was selected. It was found that the recombinant virus expressed the G protein abundantly in insect cells which was recognized by rabbit anti-rabies G polyclonal antibodies in a Western blot assay. The G protein expressed in insect cells retained its native structure as shown by immunofluorescent antibody test that the G protein expressed in insect cells reacted with 33 out of 41 monoclonal antibodies to the G protein. When lysates extracted from insect cells expressing the G protein with either 1% Triton X-100 or 1% b-octyl pyranoglucoside was given to raccoons *per os* (approximately 100 mg of G protein at day 0 and 200 mg of G protein at 7 weeks), all seven animals immunized produced neutralizing antibodies and six of them survived lethal challenge with street rabies virus while all of the seven control raccoons succumbed to rabies and died within 30 days of challenge. These data demonstrated that rabies virus G protein is a good oral immunogen and can induce neutralizing antibodies and protect immunized animals against lethal challenge when given orally.

In order to make chimeric rabies virus G protein containing HIV PND, we have used computer modeling to identify potential sites on rabies G protein to insert HIV PND sequences. It is found that amino acid residues 191 to 198, which is located in antigenic site II of the rabies G protein, is a good site for engineering HIV PND sequence. To replace amino acids 191 to 198 of rabies G protein, IFTNSRGKR (SEQ ID NO: 2), with HIV PND sequence IHIGPGRAF (SEQ ID NO: 3), site directed mutagenesis by overlapping PCR was performed with four synthetic oligonucleotides. SEQ ID NO: 4 is the 5' end of rabies G protein with the incorporation of a BgIII site and

oligonucleotide number 4 is near the 3' end of rabies G protein with the incorporation of a ClaI site. SEQ ID NOS: 5 and 6 represent the site for mutation, each has 15 nucleotides for basepairing and 27 nucleotides (coding the changed 9 amino acids) overhang. In the initial step, PCR was performed with SEQ ID NOS: 4 and 5 or SEQ ID NOS: 6 and 7 using ptg155pro8 as template. A Fragment with 0.7 kb was obtained with SEQ ID NOS: 6 and 7 and another fragment with 1.0 kb was obtained with SEQ ID NOS: 6 and 7. The corresponding fragments were collected and purified with GeneClean (Bio 101), and finally used as templates to amplify the complete chimeric gene with SEQ ID NO 7. As expected, a fragment with 1.7 kb was synthesized and this fragment will be cloned into vaccinia virus or baculovirus transfer vectors.

Oligo 1: ACTGGCAAGATCTAATATGGTTCCTCAG (SEQ ID NO:4)

Oligo 2: AAACGCTCTCCCAGGCCCAATGTGAATGTCACAAGACATCCC (SEQ ID NO:5)

Oligo 3: ATTCACATTGGGCCTGGGAGAGCGTTTGCATCCAAAGGGAGT (SEQ ID NO:6)

Oligo 4: GCGTAGGTTCTGATCGATTGACTCTTC (SEQ ID NO:7)

HIV antigens are targeted to the pregastric mucous membrane using rabies virus G protein as a delivering vehicle. The chimeric genes are constructed and expressed (rabies G-HIV PND) in vaccinia virus or baculoviruses.

Construction of chimeric gene at the discontinuous epitope site is performed as follows. As described above, the chimeric gene amplified by overlapping PCR is digested with BgIII and ClaI and cloned into ptg155pro8 also digested with the same enzymes. Alter ligation and transformation, the chimeric gene is sequenced using the primer extension method to make sure that the amplified gene has the correct HIV PND sequence in frame. The chimeric gene in the resulting clone has the complete coding sequence for rabies virus G protein within two BgIII sites as the original ptg155pro8.

Construction of chimeric genes at the linear epitope site is performed as follows. A linear epitope was mapped to residue 253 to 275 (23 residues) of the rabies virus G protein by MAb 6-15C4, which provides a site for replacement with sequence derived from HIV PND. This epitope is located between two restriction enzyme sites on the rabies virus G protein gene, with BanI at the 5' end and AatII at the 3' end. Two chimeric genes are constructed at this site. First, this epitope on rabies G protein (23 amino acids) is replaced by exact 23 amino acids derived from the PND of HIV MN strain. The 23 amino acids on rabies G protein PPDQLVNLHDFRSDEIEHLVVEE (SEQ ID NO:8) is replaced with 23 amino acids from the PND of MN strain YNKRKRIHIGPGRAFYTTKNIIG (SEQ ID NO:9). To accomplish this, two synthetic oligonucleotides (SEQ ID NO:10 and SEQ ID NO:11) with restriction sites at each end, BanI at the 5' end and AatII at the 3' end are used. These oligonucleotides will be annealed and phosphorylated, which is then cloned into ptg155pro8 digested with BanI and AatII.

GTGCTATAACAAACGCAAGCGGATTCACATCGGGCCTGGGAGAGCCTTCTATACGACCAAA
AATATAATAGGGTTGGTCAGGAAGAGAGAGGAGTGTCTGGATGCACTAGAGTCCATCATGA
CAACCAAGTCAGTGAGTTTCAG (SEQ ID NO:10)

ACGTCTGAAACTCACTGACTTGGTTGTCATGATGGACTCTAGTGCATCCAGACACTCCTCT
CTCTTCCTGACCAACCCTATTATATTTTTGGTCGTATAGAAGGCTCTCCCAGGCCCGATGT
GAATCCGCTTGCGTTTGTTATA (SEQ ID NO:11)

Second, the linear epitope on rabies G protein is replaced by the whole V3 loop. The sequence PPDQLVNLHDFRSDEIEHLVVEELVRLRDDCLDAL (SEQ ID NO:12) of rabies virus G protein is replaced with the sequence of V3 loop of HIV-1 MN strain CTRPNYNKRKRIHIGPGRAFYTTKNIIGTMRQAHC (SEQ ID NO:13). Again this is carried out with synthetic oligonucleotides (SEQ ID NO:14 and SEQ ID NO:15) with the same restriction sites as described above.

GTGCTGTACCAGGCCTAACTATAACAAGAGAAAGAGAATTCACATTGGGCCTGGGAGAGCC
TTCTATACCACTAAGAACATTATAGGGACCATAAGACAAGCCCACTGTGAGTCCATCATGA
CAACCAAGTCAGTGAGTTTCAG (SEQ ID NO:14)

ACGTCTGAAACTCACTGACTTGGTTGTCATGATGGACTCACAGTGGGCTTGTCTTATGGTC
CCTATAATGTTCTTAGTGGTATAGAAGGCTCTCCCAGGCCCAATGTGAATTCTCTTTCTCT
TGTTATAGTTAGGCCTGGTACA3 (SEQ ID NO:15)

The chimeric genes are transformed and clones with the correct mutagenesis are selected for cloning into baculovirus or vaccinia virus transfer vectors.

Expression of chimeric genes by vaccinia virus vectors is accomplished as follows. To express these chimeric genes in vaccinia virus, the protocol used to express rabies virus G protein in vaccinia virus are followed. The chimeric genes described above are digested with BgIII and cloned into the unique BamHI site of ptg1H-Tk-P7.5. The resulting clones are screened and sequenced to select those clones with the chimeric genes in the correct orientation, which are then co-transfected with wild type vaccinia virus (Copenhagen strain) into HeLa fibroblasts. HeLa cells are infected with wide type vaccinia virus two hours before recombinant plasmids are transfected. Recombinant vaccinia virus expressing these chimeric proteins are selected by infecting TK^- 143 osteosarcoma cells in the presence of 5-bromodeoxyuridine (BrdUrd). To make sure that recombinant vaccinia viruses express the correct proteins, virus eluted from plaques selected in TH^- cells are used to infect HeLa cells in 6-well plates at the multiplicity of infection (moi) of 10 plaque forming units (pfu) per cell. Four hours after infection, cells are harvested and lysed with protein disruption buffer (2% SDS, 10% glycerol, 5% b-mercaptoethanol, 62.5 mM Tris-HCl, 0.01% bromphenol blue, pH 6.8). Alter electrophoresis on 10% SDS-PAGE, proteins are electrotransferred to nitrocellulose paper and reacted with rabbit anti-G polyconoal antibodies against rabies virus G protein, monoclonal antibody to the V3 loops of MN strain. To verify that these chimeric proteins expressed by these

recombinant vaccinia viruses retain the native structure of rabies virus G protein and expressed on the cell surface, immunofluorescence antibody assays are used with monoclonal antibodies recognizing the native rabies virus G protein. In all these assays, recombinant vaccinia viruses expressing rabies glycoprotein and recombinant vaccinia virus expressing HIV gp160 are included as positive controls.

The chimeric genes are expressed in insect cells via baculovirus vectors as follows. To express these chimeric proteins in insect-cells via baculovirus vectors, the chimeric genes are digested with BgIII and cloned into the unique BamHI site of pVL941. The resulting clones are screened and sequenced to select one clone with the chimeric gene in the correct orientation in relation to the polyhedron promoter, which will then be co-transfected with AcNPV DNA into Sf9 cells. The method of limited dilution, combined with DNA dot-blot hybridization is used to detect and select recombinant baculovirus containing these chimeric genes. The recombinant baculoviruses are purified by plaque purification and used for the production of recombinant baculovirus stocks. The recombinant baculoviruses containing these chimeric genes are used to infect Sf9 cells in a 6-well plate at moi of approximately 10 pfu per cell. Infected cells are harvested at 60 hours post infection, lysed with protein disruption buffer and subjected to electrophoresis on a 10% polyacrylamide gel containing SDS. Proteins separated on the gel are electrotransferred to nitrocellulose paper and reacted with antibodies to rabies virus G protein, antibodies to V3 loop of MN strain, or sera from HIV-positive patients. Immunofluorescent antibody assays are also used to make sure that the chimeric G proteins expressed in insect cells retain its native structure. Rabies virus G protein expressed in insect cells and gp160 of HIV are included as positive controls.

TABLE I

Comparison of the Reactivity Pattern of ERA and BRG-Produced Glucoprotein with mAb in an Immunofluorescence Test

mAb	*Antigenic Site*	*Virus*	
		ERA	*BRG*
509-6	I	+++	+++
231-2	IIA	++	+
220-8		-	-
1119-14		+++	+
1107-1		+++	+++
101-1	IIB	++	++
162-3		+++	+++
1116-1		+++	+++
1121-2		++++	+++
1111-1		+++	+++
1112-1	IIC	+++	++++
613-2		+++	+++
1117-8		+	+
240-3		-	-
719-3		-	-
226-1		-	-
194-2	IIIA	+	+
248-8		+	++
523-11	IIIB	++++	+++
1105-3		++++	++++
1113-1		+++	+++
1122-3		+++	++
718-4		+++	++
1109-3		+++	+++
1114-2		++++	++++
507-1		+++	+++
120-6		++++	+++
1103-1		+++	+++
127-5		+++	+++
904-4		++	+++
1108-1	UNCL	+++	+++
176-2		++++	++++
193-2		++++	++++
504-1		-	-
508-9		-	-
419-2		-	-
422-2		-	-
1118-6		++++	++++
1120-10		+++	+++
Ab4		++++	++++
Ab5		+++	+++

TABLE 2

Oral Immunization of of Raccoons:
Baculovirus-Rabies Glycoprotein Vaccine

	Virus Neutralizing Titer (day)						
Animal #	*0*	*30*	*45*	*60*	*90*	*150*	*Survivorship*
11	5	15	5	45	15	15	S
23	5	45	15	45	15	15	ND
27	5	15	15	405	135	135	S
29	5	45	45	45	15	15	S
15	5	135	45	135	45	45	S
39	5	15	5	45	5	5	S
61	5	15	5	15	5	5	D
71	5	45	15	135	405	135	S
Conrols (n=7)	≤5	--	--	--	--	≤5	0/7

Raccoons were given 100 µg of Baculo-G vaccine *per os* on day 0, were boosted with 200 µg *per os* at 7 weeks, and were challenged with raccoon rabies virus intramuscularly on day 150 'S. survived; ND. not done (succumbed under sedation during bleeding prior to challenge); D. died

SEQUENCE LISTING

(1) GENERAL INFORMATION:
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(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WordPerfect 5.1

(vi) CURRENT APPLICATION DATA:
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(2) INFORMATION FOR SEQ ID NO:1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly Pro Gly Arg Ala Phe
1 5

(2) INFORMATION FOR SEQ ID NO:2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ile Phe Thr Asn Ser Arg Gly Lys Arg
1 5

(2) INFORMATION FOR SEQ ID NO:3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ile His Ile Gly Pro Gly Arg Ala Phe
1 5

(2) INFORMATION FOR SEQ ID NO:4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ACTGGCAAGA TCTAATATGG TTCCTCAG 28

(2) INFORMATION FOR SEQ ID NO:5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AAACGCTCTC CCAGGCCCAA TGTGAATGTC ACAAGACATC CC 42

(2) INFORMATION FOR SEQ ID NO:6:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATTCACATTG GGCCTGGGAG AGCGTTTGCA TCCAAAGGGA GT 42

(2) INFORMATION FOR SEQ ID NO:7:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCGTAGGTTC TGATCGATTG ACTCTTC 27

(2) INFORMATION FOR SEQ ID NO:8:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Pro Pro Asp Gln Leu Val Asn Leu His Asp Phe Arg Ser Asp Glu Ile
1 5 10 15

Glu His Leu Val Val Glu Glu
20

(2) INFORMATION FOR SEQ ID NO:9:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Tyr Asn Lys Arg Lys Arg Ile His Ile Gly Pro Gly Arg Ala Phe Tyr
1 5 10 15

Thr Thr Lys Asn Ile Ile Gly
20

(2) INFORMATION FOR SEQ ID NO:10:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 144 base pairs
(B) TYPE: nucleic acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GTGCTATAAC AAACGCAAGC GGATTCACAT CGGGCCTGGG AGAGCCTTCT ATACGACCAA 60

AAATATAATA GGGTTGGTCA GGAAGAGAGA GGAGTGTCTG GATGCACTAG AGTCCATCAT 120

GACAACCAAG TCAGTGAGTT TCAG 144

(2) INFORMATION FOR SEQ ID NO:11:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 144 base pairs
(B) TYPE: nucleic acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ACGTCTGAAA CTCACTGACT TGGTTGTCAT GATGGACTCT AGTGCATCCA GACACTCCTC 60

TCTCTTCCTG ACCAACCCTA TTATATTTTT GGTCGTATAG AAGGCTCTCC CAGGCCCGAT 120

GTGAATCCGC TTGCGTTTGT TATA 144

(2) INFORMATION FOR SEQ ID NO:12:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Pro Pro Asp Gln Leu Val Asn Leu His Asp Phe Arg Ser Asp Glu Ile
1 5 10 15

Gle His Leu Val Val Glu Glu Leu Val Arg Leu Arg Asp Asp Cys Leu
20 25 30

Asp Ala Leu
35

(2) INFORMATION FOR SEQ ID NO:13:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Cys Thr Arg Pro Asn Tyr Asn Lys Arg Lys Arg Ile His Ile Gly Pro
1 5 10 15

Gly Arg Ala Phe Tyr Thr Thr Lys Asn Ile Ile Gly Tyr Met Arg Gln
20 25 30

Ala His Cys
35

(2) INFORMATION FOR SEQ ID NO:14:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 144 base pairs
(B) TYPE: nucleic acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTGCTGTACC AGGCCTAACT ATAACAAGAG AAAGAGAATT CACATTGGGC CTGGGAGAGC 60

CTTCTATACC ACTAAGAACA TTATAGGGAC CATAAGACAA GCCCACTGTG AGTCCATCAT 120

GACAACCAAG TCAGTGAGTT TCAG 144

(2) INFORMATION FOR SEQ ID NO:15:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 144 base pairs
(B) TYPE: nucleic acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

ACGTCTGAAA CTCACTGACT TGGTTGTCAT GATGGACTCA CAGTGGGCTT GTCTTATGGT 60

CCCTATAATG TTCTTAGTGG TATAGAAGGC TCTCCCAGGC CCAATGTGAA TTCTCTTTCT 120

CTTGTTATAG TTAGGCCTGG TACA 144

Claims

1. An oral anti-rabies vaccine comprising at least 100 μg of an isolated rabies glycoprotein G that is produced in eukaryotic cells; and an carrier suitable for oral administration.

2. The oral anti-rabies vaccine of claim 1 comprising at least 300 μg of said isolated rabies glycoprotein G.

3. The oral anti-rabies vaccine of claim 1 wherein said isolated rabies glycoprotein G is produced in a baculovirus expression system.

4. The oral anti-rabies vaccine of claim 1 wherein said isolated rabies glycoprotein G is part of an encapsulated composition.

5. The oral anti-rabies vaccine of claim 1 further comprising food.

6. A method of immunizing a mammal against rabies infection comprising the step of orally administering to said mammal the oral anti-rabies vaccine of claim 1.

7. The method of claim 6 wherein said mammal is a raccoon.

8. The method of claim 6 wherein said isolated rabies glycoprotein G is produced in a baculovirus expression system.

9. The method of claim 6 wherein said isolated rabies glycoprotein G is administered as an encapsulated composition.

10. The method of claim 6 wherein said mammal is administered at least 300 μg of isolated rabies glycoprotein G.

11. A chimeric protein that is a modified rabies glycoprotein G comprising native rabies glycoprotein G amino acid sequence except with a 6-30 amino acid sequence substitution between amino acids 20 and 450 of said rabies glycoprotein G amino acid sequence,

said 6-30 amino acid substitution comprising an amino acid sequence that constitutes a neutralizing epitope of a pathogen.

12. The chimeric protein of claim 11 wherein said 6-30 amino acid substitution is between amino acids 175-225 of said rabies glycoprotein G.

13. The chimeric protein of claim 11 wherein said 6-30 amino acid substitution is between amino acids 150-275 of said rabies glycoprotein G.

14. The chimeric protein of claim 11 wherein said 6-30 amino acid substitution is a 9 amino acid substitution.

15. The chimeric protein of claim 14 wherein said 9 amino acid substitution is between amino acids 191-198 of said rabies glycoprotein G.

16. The chimeric protein of claim 11 wherein said 6-30 amino acid substitution is a 23 amino acid substitution.

17. The chimeric protein of claim 16 wherein said 23 amino acid substitution is between amino acids 253-275 of said rabies glycoprotein G.

18. An orally active vaccine for immunizing a mammal against a pathogen infection comprising a chimeric protein of claim 11.

19. A method of immunizing a mammal against a pathogen infection comprising the step of orally administering to said mammal at least 100 μg of a chimeric protein of claim 11.

20. The method of claim 19 wherein said mammal is a human.

21. The method of claim 19 wherein at least 300 μg of said chimeric protein is orally administering to said mammal.

22. An oral anti-pathogen vaccine comprising

at least 100 μg of an isolated rabies glycoprotein G that is produced in eukaryotic cells;

at least 100 μg of an isolated pathogen antigen or a peptide which comprises an amino acid sequence that constitutes a neutralizing epitope of said pathogen; and

an carrier suitable for oral administration.

23. The oral anti-pathogen vaccine of claim 22 comprising at least 300 μg of said isolated rabies glycoprotein G.

24. The oral anti-pathogen vaccine of claim 22 comprising at least 300 μg of an isolated pathogen antigen or a peptide which comprises an amino acid sequence that constitutes a neutralizing epitope of said pathogen.

25. The oral anti-pathogen vaccine of claim 22 comprising at least 300 μg of said isolated rabies glycoprotein G and at least 300 μg of an isolated pathogen antigen or a peptide which comprises an amino acid sequence that constitutes a neutralizing epitope of said pathogen.

26. The oral anti-pathogen vaccine of claim 22 wherein said isolated rabies glycoprotein G is produced in a baculovirus expression system.

27. The oral anti-pathogen vaccine of claim 22 comprising

at least 100 μg of an isolated rabies glycoprotein G that is produced in eukaryotic cells;

at least 100 μg of an isolated pathogen antigen; and

an carrier suitable for oral administration.

28. The oral anti-pathogen vaccine of claim 27 wherein said isolated rabies glycoprotein G is covalently linked to said isolated pathogen antigen.

29. The oral anti-pathogen vaccine of claim 22 comprising

at least 100 μg of an isolated rabies glycoprotein G that is produced in eukaryotic cells;

at least 100 μg of a peptide which comprises an amino acid sequence that constitutes a neutralizing epitope of said pathogen;

and

a carrier suitable for oral administration.

30. The oral anti-pathogen vaccine of claim 27 wherein said isolated rabies glycoprotein G is covalently linked to said peptide which comprises an amino acid sequence that constitutes a neutralizing epitope of said pathogen.

31. A method of immunizing a mammal against a pathogen infection comprising the step of orally administering to said mammal the oral anti-pathogen vaccine of claim 27 .

32. The method of claim 31 wherein said mammal is a human.

INTERNATIONAL SEARCH REPORT

In. ational application No.
PCT/US94/11112

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :424/184.1, 196.11, 224.1, 229.1, 278.1; 435/235.1, 173.1, 5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/184.1, 196.11, 224.1, 229.1, 278.1; 435/235.1, 173.1, 5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, CA, BIOSIS, AGRICOLA, CRIS/USDA, AGRIS INTERNATIONAL, CAB ABSTRACTS, PHIND, PHARMACEUTICAL NEWS INDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Proc. Natl. Acad. Sci. USA, Volume 81, issued November 1984, T. J. Wiktor et al., "Protection from rabies by a vaccinia virus recombinant containing the rabies virus glycoprotein gene", pages 7194-7198, see entire document.	1-10, 22-32
A	Reviews of Infectious Diseases, Volume 10, Supplement 4, issued November-December 1988, G. M. Baer, "Oral Rabies Vaccination: An Overview", pages S644- S648, see entire document.	1-32
A	G. M. Baer, "The Natural History of Rabies", Second Edition, published 1991 by CRC Press (Boca Raton, Florida), pages 485-503, see entire document.	1-32

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 19 DECEMBER 1994	Date of mailing of the international search report 13 JAN 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer NITA M. MINNIFIED Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

In. ational application No.
PCT/US94/11112

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Nature, Volume 322, issued 24 July 1986, J. Blancou et al., "Oral vaccination of the fox against rabies using a live recombinant vaccinia virus", pages 373-375, see entire document.	1-32
Y	J. Gen. Virol., Volume 40, issued 1978, B. Dietzschold et al., "Isolation and Purification of a Polymeric Form of the Glycoprotein of Rabies Virus", pages 131-139, see entire document.	1-32
Y	J. Wildlife Diseases, Volume 28, Number 4, issued 1992, C. E. Rupprecht et al., "Consideration of Inactivated Rabies Vaccines as Oral Immunogens of Wild Carnivores", pages 629-635, see entire document.	1-10, 22-32
X	Virology, Volume 165, Number 1, issued 1988, J. J. Esposito et al., "Successful Oral Rabies Vaccination of Raccoons with Raccoon Poxvirus Recombinants Expressing Rabies Virus Glycoprotein", pages 313-316, see entire document.	1-10, 22-32
Y	Reviews of Infectious Diseases, Volume 10, Supplement 4, issued November-December 1988, C. E. Rupprecht et al., "Efficacy of a Vaccinia-Rabies Glycoprotein Recombinant Virus Vaccine in Raccoons (*Procyon lotor*)", pages S803-S809, see entire document.	1-10, 22-32
Y	US, A, 4,650,673 (JOHNSTON ET AL.) 17 March 1987, abstract, col. 2, lines 18-33, col. 1, lines 10-55, see examples.	1-10, 22-32
P, Y	US, A, 5,266,313 (ESPOSITO ET AL.) 30 November 1993, abstract, col. 2, lines 54-70.	1-10, 22-32
Y	Proc. Natl. Acad. Sci. USA, Volume 83, issued October 1986, C. E. Rupprecht et al., "Oral immunization and protection of raccoons (*Procyon lotor*) with a vaccinia-rabies glycoprotein recombinant virus vaccine", pages 7947-7950, see entire document.	1-10, 22-32
Y	Virology, Volume 173, issued 1989, C. Prehaud et al., "Immunogenic and Protective Properties of Rabies Virus Glycoprotein Expressed by Baculovirus Vectors", pages 390-399, see entire document.	1-10, 22-32

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/11112

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proc. Natl. Acad. Sci. USA, Volume 84, issued December 1987, B. Dietzschold et al., "Induction of protective immunity against rabies by immunization with rabies virus ribonucleoprotein", pages 9165-9169, see entire document.	1-10, 22-32
Y	US, A, 5,169,784 (SUMMERS ET AL.) 08 December 1992, abstract, col. 1, lines 46-50, col. 5, lines 41-45, col. 7, lines 22-35, see examples.	1-32
Y	US, A, 5,008,373 (KINGSMAN ET AL.) 16 April 1991, col. 4, lines 19-63, see entire document.	1-32
P, Y	US, A, 5,348,741 (ESPOSITO ET AL.) 20 September 1994, see entire document.	1-32
A	US, A, 4,707,356 (PATRICK ET AL.) 17 November 1987, see entire document.	1-32
Y	WO, A, 85/01,516 (LATHE ET AL.) 11 April 1985, see entire document.	1-10, 22-32